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Abstract

Small teleost fish are increasingly used for studying the genetic basis of vision. In particular, zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) are commonly used vertebrate model organisms in developmental research, including research on the development of visual function. A multitude of behavior-based visual tests are established for larvae that have been successfully used to identify and characterize visual defects in genetically manipulated strains of these species. Testing the visual system of adult fish has proven to be more difficult for a number of reasons, including complications in restraining fish, or shoaling and dominance behavior interfering with visual behavior in population screening assays. In this paper, we present a simple and cost-effective method to quantitatively measure the optokinetic response (OKR) of individual adult zebrafish and medaka, which can be used to characterize visual capabilities of adult fish. This method can be applied to any fish species of similar size.

Introduction

Zebrafish (*Danio rerio*) as well as medaka (*Oryzias latipes*) have emerged as powerful model organisms in developmental biology. Their combination of high fecundity, extracorporally developing transparent embryos, and a large tool kit of embryological and genetic methods are used by a growing number of researchers around the world. These species are highly visual animals already as larvae, reflected by the rapid maturation of their visual system. The retina is cone dominant during all stages of development. All these properties make these two small teleosts ideal model organisms to study genetic aspects of eye development and vision (Fadool and Dowling, 2008).

Robust behavioral assays are the prerequisites to isolate and characterize genetically modified animals with visual deficits. Several behavior-based assays are currently used to assess visual capabilities of zebrafish larvae, some of which have also been applied to young medaka fish. Common behavioral tests that were already used to investigate the visual system of zebrafish larvae or to isolate recessive mutations in large-scale, forward genetic screens include the optokinetic and the optomotor response (e.g. Brockerhoff et al., 1997; Gross et al., 2005; Muto et al., 2005; Neuhauss et al., 1999). These assays are both robust and therefore well suited for high-throughput screening of larvae.

The optokinetic response (OKR) has proven to be particularly useful for testing vision of zebrafish larvae (Beck et al., 2004; Brockerhoff et al., 1997; Gross et al., 2005; Muto et al., 2005; Neuhauss et al., 1999; Rinner et al., 2005). The OKR is a stereotyped eye movement elicited by whole-field motion of the visual surround. It consists of two parts: a smooth pursuit movement in the direction of the perceived motion (OKR slow phase) and, after the eyes have reached the maximal deflection angle, a fast resetting movement in the opposite direction (saccade). The OKR is a reflexive behavior innate to all vertebrates. It depends on a simple neuronal circuit and can be elicited independently of the optic tectum or (in mammals) the cerebral cortex. Since it can be reliably elicited and lends itself to automation and quantification it is a useful tool to probe visual system properties by varying stimulus parameters. This allows assessment of contrast sensitivity, visual acuity and temporal resolution of the visual system or to probe for chromatic inputs to motion detection. To measure an OKR in larval fish, larvae are typically embedded in viscous methylcellulose solution to prevent body movement with minimal impact on eye movements (Brockerhoff, 2006; Rinner et al., 2005). Before the formation of scales, larvae are sufficiently oxygenated through their skin, rendering any life supporting measures unnecessary.

Visual behavior assays are performed with great success in a number of laboratories, particularly on genetically manipulated larvae. This is in contrast to assays testing visual behavior in the adult. The need for such assays have been low, since there are very few adult mutant strains of potential interest for vision research available. This situation is about to change with the advent of generating mutant strains with defined genetic lesions, either by TILLING (Targeting Induced Local Lesions IN Genomes) (Moens et al., 2008; Wienholds et al., 2003) or zinc finger nucleases (Doyon et al., 2008; Meng et al., 2008) and the growth of transgenic technology. Hence there will be an increasing need for robust visual assays of adult vision. So far, adult zebrafish vision has been studied using the optomotor (Krauss and Neumeyer, 2003) or the escape response (Li and Dowling, 1997). Both assays share the problem of lacking robustness and are difficult to quantify. Other studies have used time-consuming discrimination training experiments to assess adult zebrafish vision (Bilotta et al., 2005; Risner et al., 2006).

The OKR is also an ideal tool to investigate visual capabilities of adult fish, circumventing the shortcomings of the methods described above: it is a very robust response, no prior training is needed, it can be quantified, and easily automated. The main technical hurdle for measuring the optokinetic response of adult fish is to restrain body movements. Adult fish cannot be embedded in methylcellulose like larvae, since they need a constant flow of oxygenated water irrigating their gills. Therefore a method using purely mechanical restriction would be of value.

In this paper, we present such a method for measuring the optokinetic response of adult zebrafish and medaka. The animals tolerate this treatment well, so that the same individual fish can be measured at different time points, for instance before and after pharmacological or surgical treatment. Moreover, the fish can still be used for any other additional experiment and mating. Optokinetic response measurements of adult fish provide a quantifiable response, allowing the evaluation of contrast sensitivity, visual acuity and temporal resolution of adult fish. We demonstrate this by comparing the visual response of zebrafish and medaka during different stimulus conditions, such as spatial frequency, contrast and stimulus direction.

Materials and Methods

Adult zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) are briefly anesthetized in 300 mg/l MS-222 (*Sigma-Aldrich*, Switzerland) dissolved in water from their housing system (fish water). The body of the anesthetized fish is then gently clamped between two pieces of sponge, leaving the head with the eyes and gills free. The pieces of sponge are stabilized by two halves of a plastic pipe and the restrained fish, together with the pieces of sponge and the plastic half pipes, is fitted into a custom-made glass chamber (W×H×L = 12mm×12mm×65mm; Fig. 1B). A constant flow of fish water is directed straight on its gills through two inlets attached to both sides of the glass chamber. We used a maximal flow-rate of 40 ml/min on each side, which was generated by a simple peristaltic pump (SR25, 65 rpm, 24 V DC, novoprene tube N 4.8×1.6 mm, *Gardner Denver Thomas*, USA). The flow-rate of the pump can be controlled by means of a pulse-width modulator (PWM83, *National Control Devices*, USA) connected to the serial port of the control computer. The pulse-width modulator also allows shutting down the pump completely while fitting a fish into the chamber. The water effuses from the chamber at its rear end through a third tube and is directed back to the supply tank, such that a closed water cycle is formed. The water in the supply tank is maintained at a constant temperature of 28 °C using a water bath equipped with a standard aquarium heater (50 W, *Jäger*, Germany) and oxygenated by an air pump (R301, *Rena*, USA).
The flow-through chamber is placed under a dissecting microscope (SZH-10, *Olympus Corporation*, Japan), to which an infrared-sensitive CCD-camera (Guppy F-038B NIR, *Allied Vision Technologies*, Germany) equipped with an infrared-pass filter (RG 715, *Olympus Corporation*, Japan) is attached. The fish in the flow-through chamber is illuminated from below with a cluster of 15 infrared-emitting diodes (λ_{peak}=940 nm, BL0106-15-28, *Kingbright*, Taiwan) shielded by a diffusor.
A white paper drum (d=9 cm) with three small openings at the bottom edge, two for the water-supply tubes and one for the effluent tube, is placed around the fish in the flow-through chamber. A glass plate between infrared illumination and flow-through chamber serves as a stand for the paper drum (Fig. 1A).
For generating the visual stimulation, a stimulus computer is running the open-source Python library "Vision Egg" (Straw, 2008), which serves as a high level interface between Python and OpenGL. Using this library, we generate a rotating windmill-

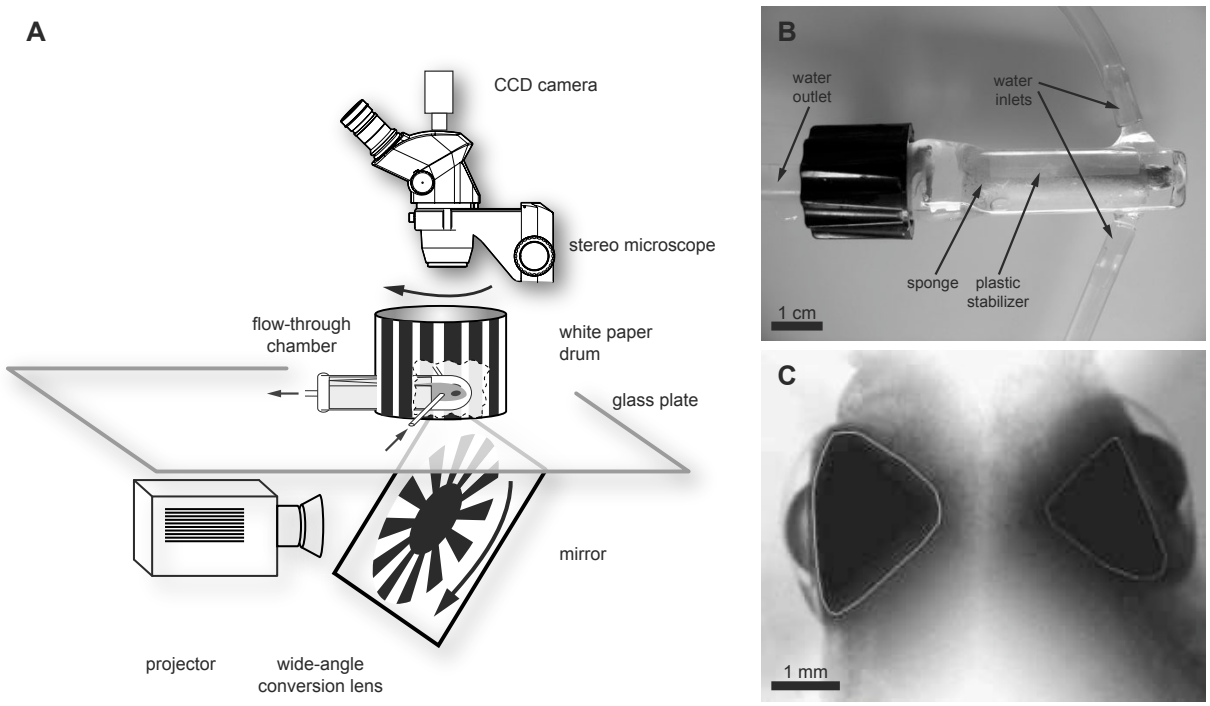

Figure 1: A) Schematic drawing of the apparatus used to measure the optokinetic response of adult zebrafish and medaka. B) Custom-made flow-through chamber with restrained zebrafish inside. C) Still-image of a movie recorded during a measurement, showing virtual white triangles overlaid on acquired image (from bottom and top) and detection of both eyes based on pixel intensity.

pattern consisting of black and white blades with diffuse borders in-between. This pattern is projected by an LCD projector (VPL-CX1, *Sony Corporation*, Japan) through a wide-angle conversion lens (HD-4500PRO, *Raynox*, Japan). The image is then deflected by a mirror oriented at 45° to the light path and projected from below onto the inside of the paper drum (Fig. 1A). By this projection, a vertical, sinusoidal grating pattern surrounding and rotating around the fish is generated. In comparison to a rotating striped drum, as used for evoking an OKR in zebrafish larvae e.g. by Brockerhoff (2006), this method of projection provides the great flexibility of computer-generated stimuli without the downsides arising when projecting a vertical stripe-pattern onto a curved screen, used by Rinner et al. (2005) (i.e. distortions and unfocused images in some areas, restriction of the stimulus to only part of the visual field).

Custom-made software based on LabView 7.1 and NI-IMAQ 3.7 (*National Instruments*, USA) is running on the control computer and controls timing as well as properties of the visual stimulation. At the same time, the software processes the images from the camera at 12.5 frames per second and recognizes the eyes of the fish based on pixel intensity. Since this operation is impeded by the dark body pigmentation of adult fish, virtual white triangles are overlaid on the acquired images to lighten up dark body parts (Fig. 1C). The size of these triangles can be varied depending on size and position of the eyes. For each eye, the software extracts the angular position and calculates the velocity in real time.

After a completed measurement, raw eye velocities (Fig. 2B) calculated from angular positions of the eyes (Fig. 2A) are processed in a similar way as described by Rinner et al. (2005). The first step in processing eye velocities is filtering out saccadic movements: Frames with an eye velocity exceeding a certain threshold are excluded from further analysis (Fig. 2C). After filtering saccades, the velocity curve is smoothened by a running average of 7 frames (Fig. 2D). In contrast to the method described by Rinner et al. (2005), we do not use a fixed threshold to filter saccades, but our program searches for an "ideal" threshold for each eye in an iterative process. This "ideal" threshold is defined as the threshold resulting in the highest overall eye velocity in the same direction as the moving stimulus after filtering.

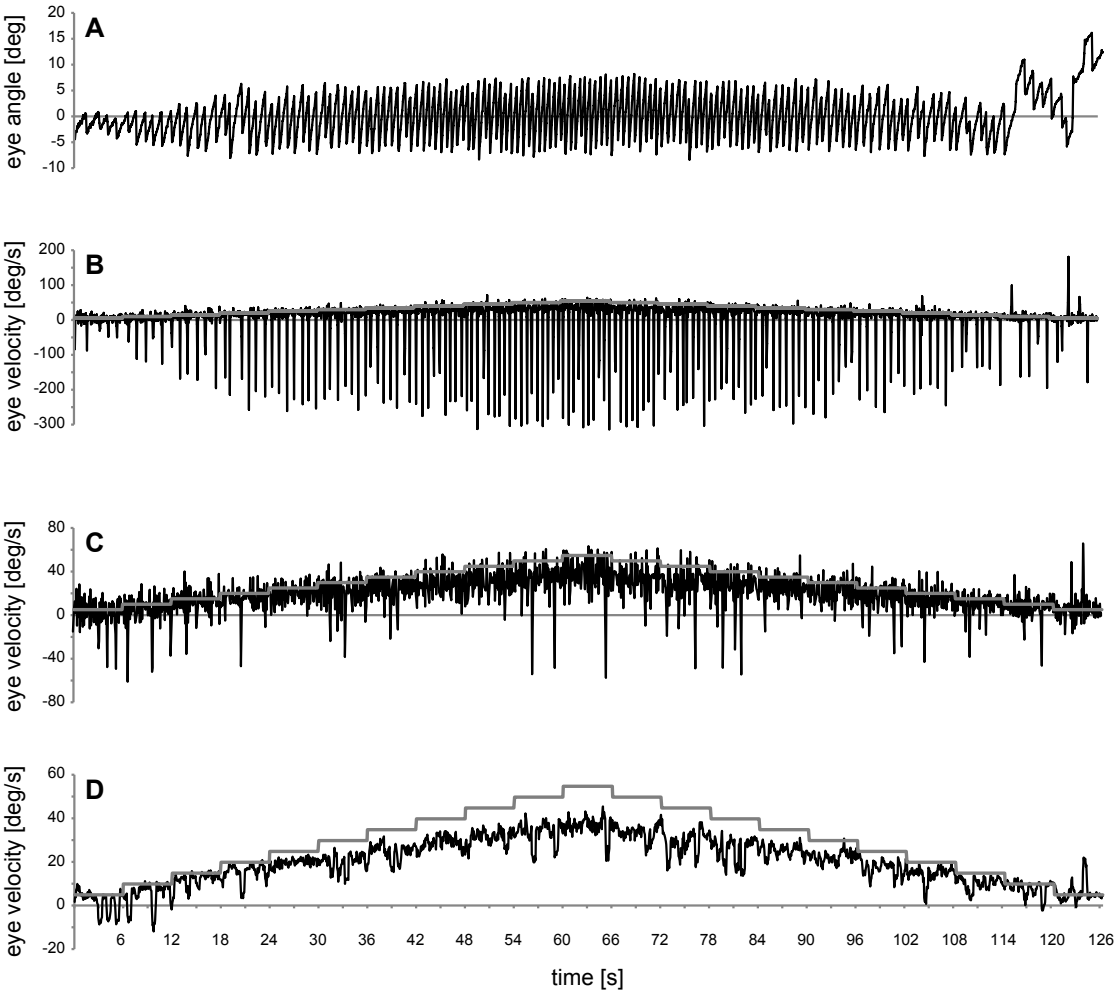

Figure 2: Example of raw measurements of the eye (black line, A) and calculated eye velocity (black line, B) of an experiment with changing angular velocity (grey line in B to D). Raw eye velocity is filtered thereupon for saccades (black line, C) and smoothened by a running average (black line, D). Note the different scaling of the y-axis in B-D.

The visual stimulation is controlled over TCP/IP allowing modification of each stimulus parameter, i.e. spatial frequency, angular velocity, orientation, contrast and color of the stripes in real time. Commands are sent via a network cable directly linking the stimulus and the control computer, ensuring absolute reliability and very high speed.

In a typical experiment, we vary only one parameter at a time. The parameter of interest is increased or reduced stepwise with steps lasting 6 s. Before the measurement of eye velocity is initiated, the eyes are pre-stimulated for 6 s with a standard stimulus (contrast = 99%, spatial frequency = 0.1 cycles·deg^{-1}, angular velocity = 12 deg·s^{-1}; contrast is normalized such that 100% denotes the maximal contrast that can be achieved).

In the case of varying contrast, we start with the highest contrast, reduce the contrast then stepwise to the lowest one and increase it again afterwards. When varying spatial frequency, we start with the lowest spatial frequency, increase it stepwise and decrease it afterwards. Similarly when varying angular velocity, we start with the lowest one (for an example, see grey line in Fig. 2D).

During one experiment, we typically stimulate only in one direction, i.e. from left to right or from right to left and evaluate one eye only, although both eyes are stimulated. This allows for more precise control of the position of the eye being evaluated, which sometimes has to be manually re-adjusted following a small movement of the fish. This readjustment can either be achieved by slightly moving the flow-through chamber manually, or, in case of very small movements, by adjusting the size of the virtual white triangles. In case of larger or prolonged movements, the measurement is aborted and re-started after the fish has calmed down, typically after few seconds. Eye velocities are averaged over each experimental condition following the process of saccade filtering and curve smoothening described above. Data was analyzed using PASW Statistics 17.0 (*SPSS Inc.*, USA); graphs were generated by R 2.9.2 (www.R-project.org).

Animal care and all experimental procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC).

Results

Optokinetic response measurements in adult zebrafish and medaka

Optokinetic response measurements were performed using two different adult (older than 12 month; n>10) inbred zebrafish strains (Tü and WIK) and the inbred medaka Cab strain. Individual fish were anesthetized and restrained as described above. We placed the fish for up to 30 minutes in the restraining device without any obvious negative effect on survival of the fish. After being released to their holding tanks they behaved indistinguishable from untreated fish.

We used a binocular stimulation paradigm with recording from one eye only. The method can be easily adapted to monocular stimulation with recording from the stimulated or unstimulated eye. For experiments probing contrast sensitivity, spatial frequency was set to 0.1 cycles·deg^{-1} and angular velocity to 12 deg·s^{-1}, varying contrast 5% to 100%. Similarly, contrast and angular velocity were held constant when spatial frequency was varied (70% and 12 deg·s^{-1}, respectively, spatial frequency ranging from 0.02 to 0.19 cycles·deg^{-1}) and contrast and spatial frequency were fixed when varying angular velocity (100% and 0.1 cycles·deg^{-1}, angular velocity between 5 and 35 deg·s^{-1}).

All stimulation paradigms resulted in robust responses with little variance between individual fishes and different experiments using the same fish, in both fish species. We also found no significant differences between the two different zebrafish strains (repeated measures ANOVA, direction of stimulation and contrast, spatial frequency or angular velocity, respectively, as within-subject effects, fish strain as between-subject effect. Contrast: $F_{1,21} = 3.073$, $p = 0.094$; Spatial frequency: $F_{1,21} = 1.389$, $p = 0.252$; Temporal frequency: $F_{1,21} = 1.382$, $p = 0.253$). As a proof of principle we then used this method to measure directional asymmetry of the optokinetic reflex and compared visual response of zebrafish and medaka.

Directional asymmetry in the optokinetic response of adult zebrafish

In order to quantify directional asymmetry, we measured left eye velocity depending on contrast, spatial frequency and angular velocity of the stimulation for both directions of movement separately. We observed a clear directional asymmetry in the optokinetic response of adult zebrafish: Eye velocities in nasal-to-temporal direction were significantly lower than in temporal-to-nasal direction under all experimental conditions (Fig. 3; repeated measures ANOVA, direction of stimulation and contrast, spatial frequency or angular velocity, respectively, as within-subject effects; contrast: $F_{1,22} = 142.556$, $p < 0.001$; spatial frequency: $F_{1,22} = 130.763$, $p < 0.001$; angular velocity: $F_{1,22} = 116.146$, $p < 0.001$). In nasotemporal direction, our method

of saccade filtering on average even resulted in negative eye velocities for low contrasts and low spatial frequencies. This artefact can be explained by the fact that slow eye movements of zebrafish in nasotemporal direction are not very smooth, especially under low contrasts or low spatial frequencies stimulus conditions. Under these conditions, pursuit movements in nasotemporal direction and saccades are of almost the same velocity, occasionally pursuit movements are even faster than resetting movements, leading to the impression that the eyes are "jumping" back and forth. As a consequence of our filtering method, these peculiar eye movements then may result in negative overall-velocities. However, with higher contrasts and spatial frequencies, this artefact is not apparent and our filtering method results in reliable slow-phase velocities.
A certain danger may lie in our method of searching the "optimal" threshold for saccade filtering: When measuring fish with visual defects, this method could lead to totally different thresholds for affected and unaffected individuals. In this case, it may prove safer to use a fixed threshold. For healthy individuals, using a fixed threshold does not qualitatively change the results, but only leads to slightly decreased eye velocities (Suppl. Figs. S1 and S2).

Comparing eye velocities of adult zebrafish and medaka

In order to compare the visual responses of medaka and zebrafish in the optokinetic response paradigm, we used binocular stimulation, evaluating only the eye stimulated in temporal-to-nasal direction.

Contrast sensitivity:

We measured contrast sensitivity of both the zebrafish inbred strains (WIK (n=14) and Tü (n=12)) and medaka inbred Cab strain (n=23). Since they showed no significant difference, we combined the data of the zebrafish strains for the comparative analysis. Spatial frequency and angular velocity were fixed to 0.1 cycles·deg^{-1} and 12 deg·s^{-1}, respectively; contrast was varied between 1% and 100%. Overall, eye velocity of zebrafish and medaka did not differ significantly (Fig. 4A; repeated measures ANOVA, contrast as within-subjects effect, fish species (zebrafish or medaka) as between-subjects effect; $F_{1,47} = 2.749$, $p = 0.104$). However, under low contrast conditions (contrast<10%), eye velocity of medaka fish was significantly lower (e.g. at 5% contrast: two-tailed t-test, $t_{47} = -3.549$, $p < 0.001$). When measuring contrast sensitivity with higher stimulus velocity (30 deg·s^{-1}), this effect was even more pronounced, leading to a strong decline and significantly lower eye velocity of medaka already at 20% contrast (Suppl. Fig. S3A; two-tailed t-test, $t_{46} = -3.689$, $p < 0.001$). This result suggests a lower contrast sensitivity of medaka fish compared to zebrafish.

Spatial resolution:

For a comparison of the spatial resolution, we again compared medaka (Cab, n=23) and zebrafish (total n=25; n(Tü) = 14; n (WIK) = 11). Contrast and angular velocity were held constant at 70% and 12 deg·s^{-1}, respectively, whereas spatial frequency was varied in the range from 0.04 to 0.35 cycles·deg^{-1}. Again, there is no significant overall difference between zebrafish and medaka (Fig. 4B; repeated measures ANOVA, spatial frequency as within-subjects effect, fish species as between-subjects effect; $F_{1,46} = 2.655$, $p = 0.110$). At higher spatial frequencies (>0.2 cycles·deg^{-1}), eye velocity of medaka fish is significantly lower (e.g. at 0.23 cycles·deg^{-1}: two-tailed t-test, $t_{46} = -2.506$, $p = 0.016$) and, again, this effect is much stronger when measuring with higher stimulus velocity, where the curve for medaka fish rapidly drops off for spatial frequencies above 0.12 cycles·deg^{-1} (Suppl. Fig. S3B). This result implies that spatial resolution of medaka fish is lower compared to zebrafish.

Temporal resolution:

To investigate temporal resolution, we measured the optokinetic response under varying angular velocity (ranging from 5 to 50 deg·s^{-1}) (n=23 (medaka Cab); n=27 (zebrafish; 14 WIK; 13 Tü). Contrast was held constant at 100%; likewise, spatial frequency was set to 0.1 cycles·deg^{-1}. We found no difference in temporal resolution between zebrafish and medaka (Fig. 4C; repeated measures ANOVA, angular velocity as within-subjects effect, fish species as between-subjects effect; $F_{1,48} = 0.000$, $p = 0.983$). Only at the lowest stimulus velocity (5 deg·s^{-1}), eye velocity of medaka was significantly lower (two-tailed t-test, $t_{48} = -2.890$, $p = 0.006$).

Discussion

In the present report we describe a quick and simple method to quantitatively record eye movements in adult medaka and zebrafish. In principal this methods should be applicable to any small aquatic species that fits into our holding chamber.

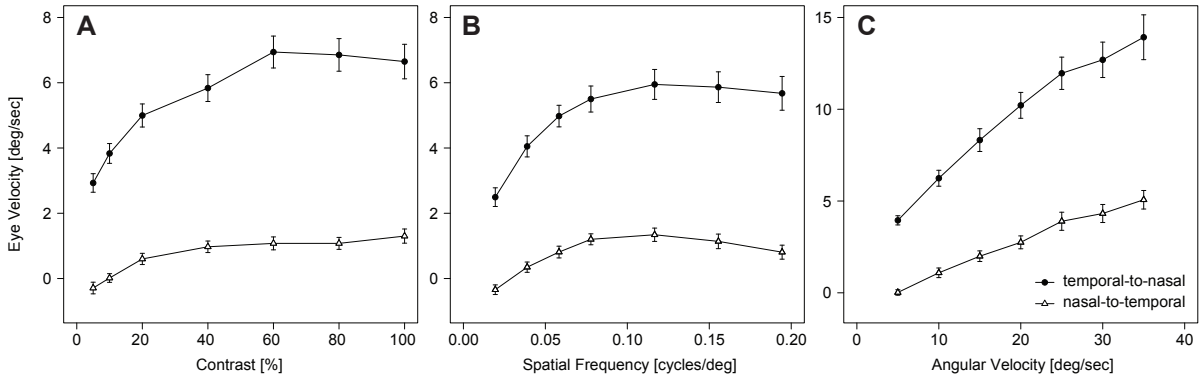

Figure 3: Averaged left eye velocity of adult zebrafish under temporal-to-nasal and nasal-to-temporal stimulation with changing contrast (A), spatial frequency (B) and angular velocity (C). Error bars indicate ±1 standard error.

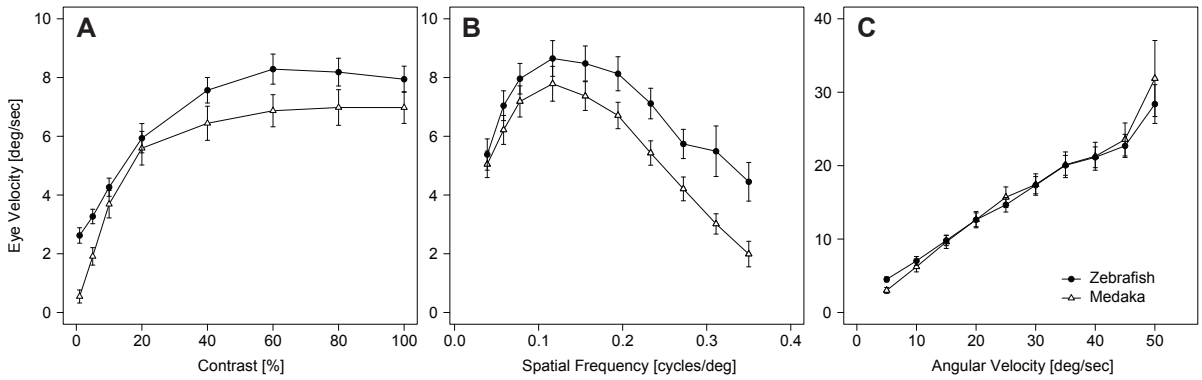

Figure 4: Averaged temporal-to-nasal eye velocity of adult zebrafish and medaka with changing contrast (A), spatial frequency (B) and angular velocity (C). Error bars indicate ±1 standard error.

Since the animals were only mechanically restrained and are supported by oxygenated water flushing their gills, the set-up is ideally suited to spike the water with chemical to study the influence of pharmacological agents on eye movements. Eye movements were recorded by a CCD camera and analyzed in real time with a custom-made software. Fish tolerated the experiment very well and stable eye movements could be recorded after stimulation with moving objects projected by a LCD projector, without any harm to the animals.

In order to validate the method we tested contrast sensitivity, spatial and temporal resolution of the optokinetic response in zebrafish and medaka.

We found a strong directional asymmetry, favoring movements in the temporal-to-nasal direction. This behavioral asymmetry of adult zebrafish is in line with previous findings in larval zebrafish (Qian et al., 2005), and other lateral eyed animals, e.g. turtles (Ariel, 1990), pigeons (Gioanni et al., 1981) and chicken (Wallman and Velez, 1985). In all these animals, temporal-to-nasal eye velocity proofed to be higher than nasal-to-temporal velocity, especially under monocular stimulation.

Therefore we decided only to consider temporal-to-nasal velocities in the experiment comparing zebrafish and medaka. The bell-shaped curve for spatial resolution and also the shape of the curve for contrast sensitivity of adult zebrafish and medaka are well in agreement with the observations from larval zebrafish (Rinner et al., 2005). The linear increase of eye speed with increasing stimulus velocity is expected and can also be observed in larval fish, albeit – not surprisingly – eye movements of adult fish reach much higher velocities. A measure often used in optokinetic research is the slow phase gain (SPG), defined as eye velocity divided by stimulus velocity. The SPG we obtained with our method only reaches 1 at the lowest angular velocity of the stimulation, at higher stimulus velocities, we observed a maximal gain between 0.6 and 0.8. This observation likely supports the notion that optokinetic eye movements have evolved to track slow moving objects.

By applying our method to medaka fish, we could show that the technique is not bound to zebrafish. The method described here provides a useful tool for characterizing visual capabilities of adult zebrafish and medaka, two species commonly used as model organisms. In fact, the method should be easily applicable to any small species of fish fitting into the flow-through chamber described. All in all, visual performance of zebrafish and medaka is quite similar – a finding not surprising given the similar size, habitats and nutrition of the two species. For a trained experimenter, restraining one fish does not take longer than one minute. Together with the 1-2 minutes we usually let the fish recover from anesthesia before starting with the measurements, and the approximately 1.5 minutes (depending on the paradigm) for the measurement itself, we achieve a good characterization of the visual capabilities of one fish in less than 5 minutes. We believe that this time is sufficiently short, such that our method could be used to screen for mutations affecting the visual system of adult fish.

Acknowledgments

We like to thank Markus Tschopp for initial experiments and the suggestion of the virtual triangles. This study was supported by the European commission (RETICIRC project).

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Supplementary Figures

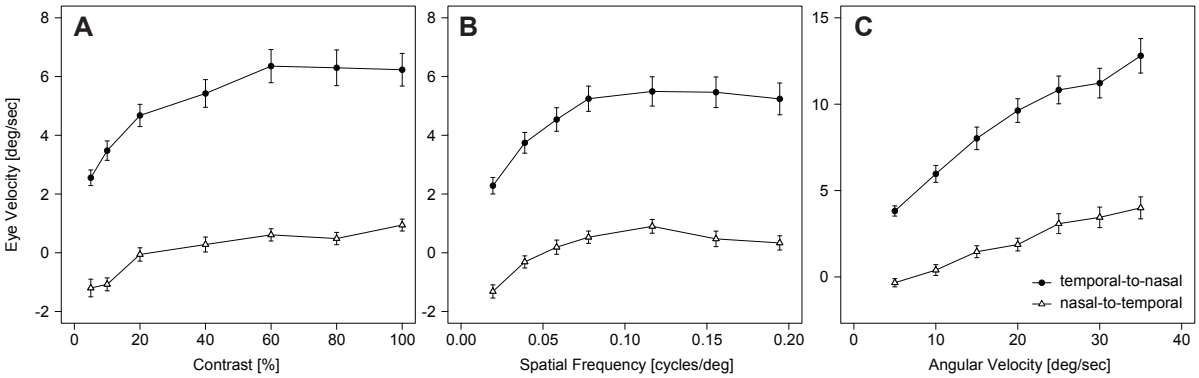

Figure S1: Averaged left eye velocity of adult zebrafish under temporal-to-nasal and nasal-to-temporal stimulation with changing contrast (A), spatial frequency (B) and angular velocity (C). Saccades were filtered using a fixed threshold of 35 deg·s^{-1}. Error bars indicate ±1 standard error.

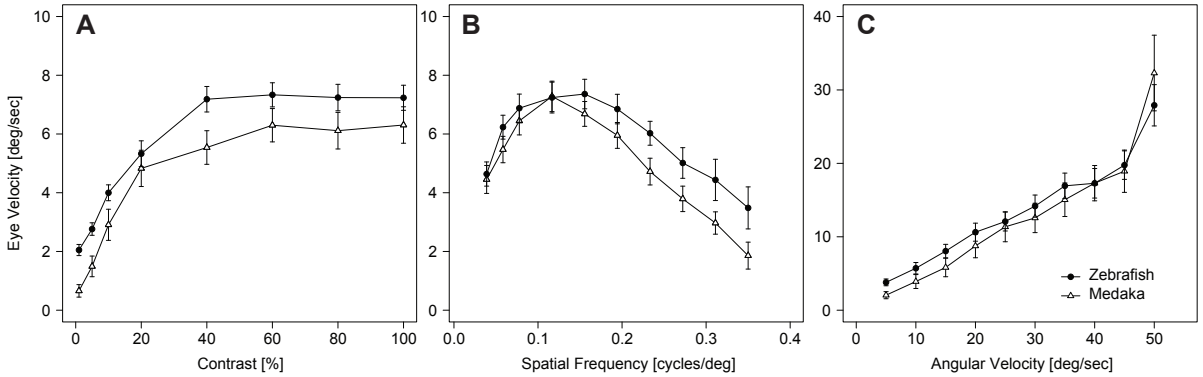

Figure S2: Averaged temporal-to-nasal eye velocity of adult zebrafish and medaka with changing contrast (A), spatial frequency (B) and angular velocity (C). Saccades were filtered using a fixed threshold of 35 deg·s^{-1} (A and B) or 100 deg·s^{-1} (C). Error bars indicate ±1 standard error.

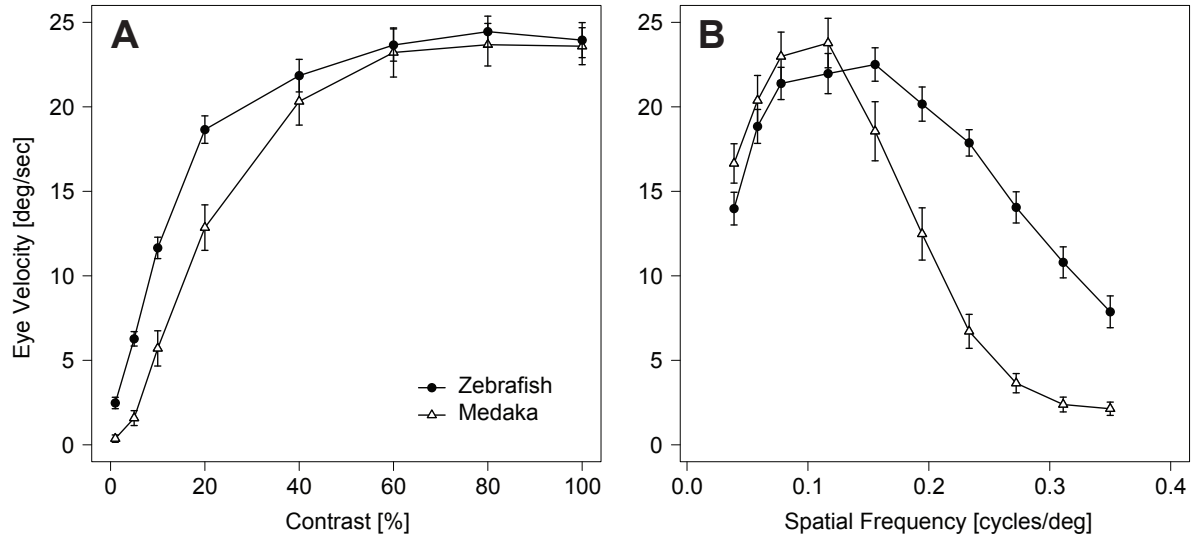

Figure S3: Averaged temporal-to-nasal eye velocity of adult zebrafish (WIK, n=24) and medaka (Cab, n=24) with changing contrast (A) and spatial frequency (B). Angular velocity of stimulation was set to 30 deg·s^{-1}, spatial frequency in A to 0.1 cycles·deg^{-1}, contrast in B to 70%. Error bars indicate ±1 standard error.